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(54) Title: SCREENING ASSAY FOR THE IDENTIFICATION OF INHIBITORS OF MACROPHAGE MIGRATION INHIBITORY FACTOR

(57) Abstract

There is disclosed assays to identify compounds that inhibit an enzymatic activity of MIF (macrophage migration inhibitory factor). The enzyme activity catalyzes a tautomerization of MIF-substrates, such as D-dopachrome to DHICA. In general, the assay is conducted *in vitro* by adding, mixing or combining MIF polypeptide and a suitable substrate in the presence or absence of a test compound, and measuring the tautomerization of the substrate. The test compounds that inhibit tautomerization in the assay are identified as MIF inhibitors.

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SCREENING ASSAY FOR THE IDENTIFICATION OF INHIBITORS OF MACROPHAGE MIGRATION INHIBITORY FACTOR

Technical Field of the Invention

The present invention provides a screening assay for identifying therapeutic inhibitors of macrophage migration inhibitory factor (MIF) biological activity. The present invention further provides therapeutic agents capable of inhibiting MIF biological activity and identified by the inventive screening assay. In particular, the therapeutic agents are useful for treating various conditions involving cytokine-mediated toxicity, such as, shock, inflammatory diseases, graft-versus-host disease, and autoimmune diseases.

BACKGROUND OF THE INVENTION

Infection by a variety of microorganisms, including not only bacteria but also viruses, fungi, and parasites, can induce septic shock. Septic shock is a multifaceted pathological condition characterized by deleterious hemodynamic changes and coagulopathy. Septic shock can lead to multiple organ failure and often to death. The shock syndrome is more properly associated with the host's response to microorganism invasion. In the case of infection by gram-negative bacteria, one of the best studied examples, it is believed that the appearance of bacterial endotoxins, such as lipopolysaccharide (LPS), in the host bloodstream leads to the endogenous production of a variety of host factors that directly and indirectly mediate the toxicity of LPS. LPS itself is relatively innocuous to most cells. These host-derived mediators include many inflammatory cytokines and classical endocrine hormones in addition to a number of other endogenous factors, such as leukotrienes and platelet activating factor. It is generally acknowledged, however, that the full cast of host-derived mediators and each of their interrelated roles in the host response remains incompletely appreciated.

In general, those host-derived mediators that appear earlier in an invaded host are thought to trigger the release of later appearing factors. Also, many endogenous mediators not only exert direct effector functions at their target tissues, but also prime local and remote tissues for subsequent responses to other mediators. This interacting network of host factors has been termed the "cytokine cascade." This term is meant to indicate the rapid extension and amplification of the host response in such a way that only one or a few initiating stimuli trigger the eventual release and participation of scores of endogenous mediators. Although a number of features of the host response are thought to assist in fighting off invasion, an overly robust or poorly modulated host response can rapidly accelerate to produce such profound alterations in host homeostasis at the cellular, tissue, and systemic levels that death may ensue within hours.

Murine macrophage migration inhibitory factor (MIF) was identified as an LPS-induced pituitary protein (Bernhagen et al., *J. Cell. Biochem. Supplement 17B*, E306, 1993). Initially, it was hypothesized that MIF might be a pituitary-derived protective factor, capable

of counteracting the adverse effects of cytokines in endotoxemias, but it subsequently was found that MIF actually exacerbates endotoxin-induced shock, and that inhibition of MIF activity can be used to treat otherwise lethal effects of cytokine-mediated shock. MIF has been re-defined to be an anterior pituitary hormone, a macrophage cytokine, and a critical component of the host response to septic shock (Bernhagen et al., *Nature* 365:756-759 1993; Calandra et al., *J. Exp. Med.* 179:1895-1902 1994; Calandra et al., *Nature* 377:68-71 1995).

MIF was first described over 30 years ago as a T cell product that inhibits the random migration of guinea pig macrophages in an *in vitro* assay (George and Vaughan, *Proc. Soc. Exp. Biol. Med.* 111:514-521 1962; Bloom and Bennett, *Science* 158:80-82 1966; David, *Proc. Natl. Acad. Sci. USA* 65:72-77 1966). MIF had been reported: (1) to be associated with delayed-type hypersensitivity reactions (Bloom and Bennett, 1966, *supra*; David, 1966, *supra*), (2) to be produced by lectin-activated T cells (Weiser et al., *J. Immunol.* 126:1958-1962 1981), and (3) to enhance macrophage adherence, phagocytosis and tumoricidal activity (Nathan et al., *J. Exp. Med.* 137:275-288 1973; Nathan et al., *J. Exp. Med.* 133:1356-1376 1971; and Churchill et al., *J. Immunol.* 115:781-785 1975). Many of these studies used mixed culture supernatants that were shown later to contain other cytokines, such as IFN-γ and IL-4, that also exhibit migration inhibitory activity (McInnes and Rennick, *J. Exp. Med.* 167:598-611 1988; Thurman et al., *J. Immunol.* 134:305-309 1985).

Recombinant human MIF was originally cloned from human T cells (Weiser et al., *Proc. Natl. Acad. Sci. USA* 86:7522-7526 1989). The biological activity profile of MIF was incompletely known and has been debated. However, MIF has been shown (1) to activate blood-derived macrophages to kill intracellular parasites and tumor cells *in vitro*, (2) to stimulate IL-1β and TNFα expression, and (3) to induce nitric oxide synthesis (reviewed in Bernhagen et al., *Biochemistry* 33:14144-14155 1994).

MIF has been described to be an anterior pituitary hormone and to be released from immune cells stimulated by low concentrations of glucocorticoids. Once secreted MIF acts to control, or counter-regulate, the immunosuppressive effects of glucocorticoids on inflammatory cytokine production and acts to modulate the potent anti-inflammatory properties of glucocorticoids which are necessarily produced as part of the host response to infection and tissue invasion (Calandra et al., *Nature* 377:68-71 1995). MIF is a critical component of the immune system and acts to counter the immunosuppressive effect of glucocorticoids to regulate inflammation and immunity.

In contrast to MIF's expected function as a protective hypothalamic factor, MIF exacerbated lethality in several models of septic shock. Thus, a robust MIF response within the cytokine cascade may be undesired. Moreover, the lack of defined cellular and biochemical activities of MIF has made it very difficult for researchers to develop an assay to specifically measure and quantitate MIF immunologic activity and identify MIF inhibitors. The lack of an *in vitro* assay to identify MIF inhibitors has hampered the investigation of the precise biological profile of this molecule in the immune response and the development of a

treatment for disorders related to cytokine-mediated toxicity.

SUMMARY OF THE INVENTION

The present invention provides a rapid, quantitative and specific assay for screening test compounds (*e.g.*, drugs, ligands (natural or synthetic), proteins, peptides and small organic molecules) for activity to inhibit biological activity of macrophage migration inhibitory factor (MIF). The present invention also provides identified compounds (*i.e.*, drugs, ligands, proteins, peptides and small organic molecules) identified by the inventive screening assay, to be capable of inhibiting biological activities of MIF. The invention further provides a method for using such identified compounds for the treatment of various conditions involving cytokine-mediated toxicity, which include, but are not limited to shock, inflammatory diseases, graft-versus-host disease, and autoimmune diseases.

The invention is based, in part, on the surprising discovery that MIF catalyzes a tautomerization reaction, and that assays for inhibitors of this tautomerase activity can be used to identify compounds that inhibit other biological activities of MIF.

BRIEF DESCRIPTION OF THE FIGURE

Figure 1 shows a scheme for the conversion of D-dopachrome catalyzed by D-dopachrome tautomerase and by MIF.

DETAILED DESCRIPTION OF THE INVENTION

Definitions:

The following definitions are used.

"MIF" is macrophage migration inhibitory factor polypeptides having MIF activity but excluding IFN-γ and IL-4.

"MIF tautomerase activity", "MIF dopachrome tautomerase activity" and "MIF tautomerization activity" each refer to the same tautomerase enzymatic activity of MIF indicated by the ability of MIF to catalyze the tautomerization of a MIF-substrate.

"MIF substrate" includes but is not limited to (a) D-dopachrome [D-3,5-dihydro-6-hydroxy-5-oxo-2H-indole-2-carboxylic acid], (b) D-dopachrome-methyl ester [D-3,5-dihydro-6-hydroxy-5-oxo-2H-indole-2-carboxylic acid methyl ester], or (c) L-dopachrome-methyl ester [L-3,5-dihydro-6-hydroxy-5-oxo-2H-indole-2-carboxylic acid methyl ester], or derivatives thereof.

"Test compounds" or "test substances" or "test inhibitors" each refers to candidate drugs, ligands (natural or synthetic), proteins, peptides or small organic molecules to be tested for their ability to inhibit the tautomerase enzymatic activity of MIF.

Tautomerase Activity of MIF

The tautomerase enzymatic activity of MIF was uncovered during an investigation of the biochemical pathways of melanogenesis. The late stages of melanin biosynthesis involve

an enzymatic conversion of L-2-carboxy-2,3-dihydroindole-5,6-quinone (L-dopachrome) into 5,6-dihydroxyindole-2-carboxylic acid (DHICA). A distinct enzyme activity isolated from bovine eye lens catalyzes the tautomerization of D-dopachrome, the non-physiological stereoisomer of the natural compound, L-dopachrome, to DHICA (Figure 1). Purification and N-terminal sequence analysis of the protein responsible for this tautomerase activity for the non-naturally occurring substrate, D-dopachrome, identified the enzyme to be the bovine homolog of MIF. The enzymatic activity of purified, native MIF was confirmed by studies of recombinant human MIF, which was also found to catalyze selectively the same tautomerization reaction with D-dopachrome as substrate, but to be inactive toward L-dopachrome. As a result of these studies, the present invention encompasses assays to identify compounds that inhibit the enzymatic activity of MIF which catalyzes the tautomerization of MIF-substrates, such as D-dopachrome to DHICA. In general, the assay is conducted *in vitro* by adding, mixing or combining MIF and a suitable substrate in the presence or absence of a test compound, and measuring the tautomerization of the substrate. The test compounds that inhibit tautomerization activity in the inventive assay are MIF inhibitors having important pharmacologic activity.

The present invention provides the use of such identified MIF inhibitors in pharmaceutical compositions designed to inhibit MIF biological activity for the treatment of cytokine toxicity-related disorders. The present invention encompasses the preparation of such pharmaceutical compositions and methods to use such compositions for the treatment of various conditions involving cytokine-mediated toxicity, which include, but are not limited to shock, inflammatory diseases, graft-versus-host disease, and autoimmune diseases.

Assays

The present invention provides a rapid and quantitative method for screening compounds that inhibit the biological activities of MIF. The assays of the present invention involve measuring the ability of a test substance to inhibit the MIF-catalyzed tautomerization of specific MIF-substrates. The test substances or test compounds include but are not limited to drugs, ligands (natural or synthetic), proteins, peptides or small organic molecules that inhibit the ability of MIF to enzymatically catalyze the tautomerization reaction. Those test substances which are identified as inhibitors of MIF enzymatic activity, are candidates for inhibitors of MIF biological activity, including but not limited to MIF-induced inflammatory and immunological activities. The inventive screening assay encompasses measuring the effect of a test substance on the ability of MIF to catalyze the tautomerization of a specific substrate.

Specifically, the inventive assay first prepares a reaction mixture of MIF polypeptide and an MIF-substrate in the presence and absence of the test substance or test compound. Tautomerization of the MIF-substrate is measured. Compounds that inhibit MIF-catalyzed tautomerization are the candidate compounds that can be used in accordance with the invention. While any order of addition of the reaction components can be utilized, it is

preferred to add the MIF-substrate to a mixture of MIF and the test compound, or to add MIF to a mixture of the MIF-substrate and the test compound. Such order of additions would facilitate readout, since the assay reaction will not proceed until both the catalyst and substrate components are combined. The assay may be formatted as a homogeneous assay, for example, with all components in solution, or as a heterogeneous assay, for example, wherein either MIF or the MIF-substrate is immobilized on a solid support.

Reaction components and conditions that may be used in the screening assays of the invention include, but are not limited to, tautomerization reactions described in Rosengren et al., *Molecular Medicine* 2:143-149 1996, and Aroca et al., *Eur. J. Biochemistry* 208:155-163 1992, each of which is incorporated by reference herein in its entirety. Sources of MIF may include, but are not limited to, mammalian tissue (*i.e.*, human, chicken, bovine, mouse, and rat tissue), bovine tissue (*i.e.*, eye lens is a source of preformed bioactive MIF protein), and hepatocytes (an abundant source of preformed bioactive MIF protein). Preferably, recombinant MIF is used. Bernhagen et al. (*Biochemistry* 33:14144-14155 1994) describes a procedure to purify bioactive recombinant MIF.

MIF-substrates that can be used in the assay include, but are not limited to, D-dopachrome, D-dopachrome methyl ester and L-dopachrome methyl ester. D-dopachrome is the non-naturally occurring D-isomer of L-2-carboxy-2,3-dihydroindole-5,6-quinone (L-dopachrome). The methyl esters of the D- and L-isomers are better tautomerase substrates for MIF than D-dopachrome in the sense that they are much more rapidly tautomerized. These substrates are generally formed immediately prior to conducting the assay, by oxidation of the corresponding phenylalanine analog. For example, D-DOPA [or D-3-(3,4-dihydroxyphenylalanine] is oxidized to D-dopachrome [D-3,5-dihydro-6-dihydroxy-5-oxo-2H-indole-2-carboxylic acid]. In a preferred embodiment of the present invention, L-dopachrome methyl ester is used as the MIF-substrate in the screening assay. In another embodiment of the present invention, D-dopachrome methyl ester may be used as the MIF-substrate in the screening assay.

In one embodiment of the invention, a reaction mixture is prepared that contains a bioactive form of MIF in the presence or absence of a test compound, and an MIF-substrate (*e.g*, D-dopachrome). The ability of the test compound to inhibit the enzymatic activity of MIF is determined by measuring the conversion of the orange-colored D-dopachrome substrate to colorless DHICA spectrophotometrically. For example, the colored MIF substrate can be prepared immediately prior to assay by oxidizing a colorless DOPA derivative to form the colored substrate, D-dopachrome. The test compound and bioactive MIF is added to the colored MIF-substrate. Tautomerization of the MIF substrate by MIF results in a non-colored solution of DHICA and the conversion can be monitored, for example, spectrophotometrically over a defined period, for instance one minute.

The presence of a test compound which is positive for MIF inhibiting activity will inhibit the tautomerization activity of MIF. This assay results in a solution which maintains

the D-dopachrome color. Therefore, the ability of a test compound to inhibit MIF activity can be alternatively visualized colorimetrically, or quantitated spectrophotometrically. For example, the tautomerization of D-dopachrome to DHICA is readily determined spectrophotometrically by measuring the rate of decrease of the iminochrome absorbance at a wavelength of 475 nm. Another means for measuring tautomerization includes, for example, HPLC resolution of products and substrates.

In a preferred embodiment of the invention the assay of the invention involves adding a test compound to a reaction mixture containing about 0.5 mM of the MIF-substrate L-dopachrome methyl ester. This MIF-substrate is prepared immediately prior to assay by oxidation of L-DOPA methyl ester with periodate to yield the colored substrate L-dopachrome methyl ester. Approximately 50-250 ng of recombinant MIF is added to 1 ml of the substrate solution. The reaction is assayed directly in a cuvette by measuring the decrease of absorbance at 475 nm, or 550 nm for substrate concentrations higher than 0.5 mM. The resulting reaction product is the colorless compound 5,6-dihydroxyindole-2-carboxylic acid methyl ester (DHICA-ME). Test compounds which inhibit the decrease in absorbance are inhibitors of MIF D-dopachrome tautomerase activity and thereby identified as candidate inhibitors of inflammation-related or immune-related activities of MIF.

This assay offers several advantages. The assay provides a rapid, quantitative and specific means to measure the enzymatic activity of MIF. This assay provides a rapid screening procedure to identify inhibitors of the enzymatic activity of MIF. Those inhibitors identified will serve as candidate inhibitors of other biological activities of MIF, such as immunomodulatory activity.

MIF Activity Inhibitors

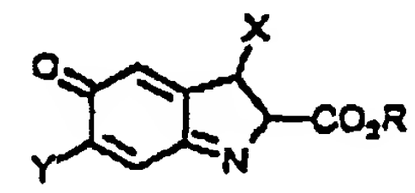
The present invention further provides drugs, ligands, proteins, peptides or small organic molecules identified as inhibitors of MIF enzymatic activity. In addition, the present invention provides methods for the use of the identified MIF inhibitors in pharmaceutical compositions to treat disorders related to cytokine-mediated toxicity, which include, but are not limited to shock, inflammatory diseases, graft-versus-host disease, and autoimmune diseases. The inhibitors also have utility in that they act in an anti-inflammatory or otherwise beneficial capacity by increasing the therapeutic efficacy of glucocorticoids, whether such glucocorticoids are present endogenously or exogenously administered.

The present invention also provides compounds, such as drugs, ligands (natural or synthetic), proteins, peptides, or small organic molecules, identified in the inventive assay as inhibitors of the biological activity of MIF. In one embodiment of the invention, certain MIF inhibitors might inhibit the tautomerization of the substrate by competing with the substrate for MIF binding. Certain of these compounds are thought to interact with MIF but not to be tautomerized due to their specific structural features. These direct competitors include, but are not limited to, the D- and L-forms of α-methyl dopachrome methyl ester. The use of this type of inhibitor of MIF also has utility in targeting the tautomerase-related substrate binding

site of MIF in order to bring a second functional element of a bi-functional inhibitor into effective molecular registration or steric alignment to inhibit the inflammatory or immunomodulatory activity of MIF.

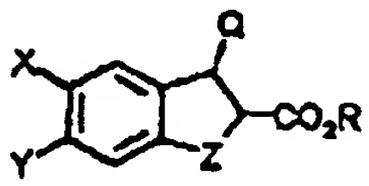
In another embodiment of the invention, certain MIF inhibitors (*e.g.*, glutathione and typanothione (which is two molecules of glutathione covalently linked by a spermidine bridge)) inhibit the tautomerization reaction by interacting with an epitope of MIF that is critical for enzymatic activity, for instance the amino terminus. The use of this type of inhibitor of MIF also has utility in targeting the amino terminus of MIF in order to bring a second functional element of a bi-functional inhibitor compound into effective molecular registration or steric alignment to inhibit the inflammatory or immunomodulatory activity of MIF.

Examples of compounds within the scope of the invention are shown below as nine series of compounds according to formulae I through IX:



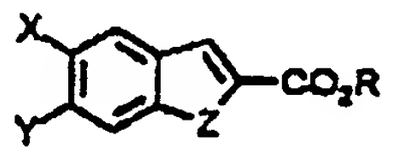
X = H, F, Cl, Br, OH, OR'
Y = H, F, Cl, Br, OH, OR'

I



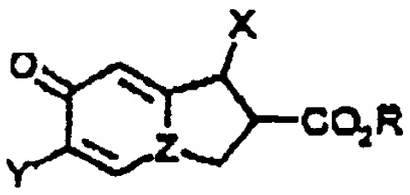
Q = H, F, Cl, Br, OH, OR'
X = H, F, Cl, Br, OH, OR'
Y = H, F, Cl, Br, OH, OR'
Z = NH, NR", CH_2, O, S, SO, SO_2

II



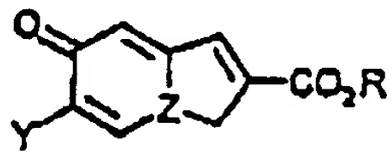
X = H, F, Cl, Br, OH, OR'
Y = H, F, Cl, Br, OH, OR'
Z = CH_2, NH, NR", O, S, SO, SO_2

III



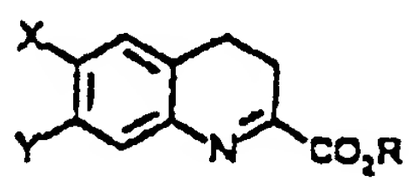
Y = H, F, Cl, Br, OH, OR'
X = H, F, Cl, Br, OH, OR'
Z = N, C-CH_3

IV



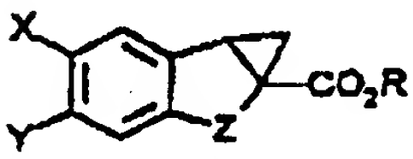
Y = H, F, Cl, Br, OH, OR'
X = H, F, Cl, Br, OH, OR'
Z = N, C-CH_3

V



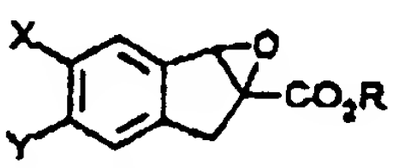
X = H, F, Cl, Br, OH, OR'
Y = H, F, Cl, Br, OH, OR'

VI



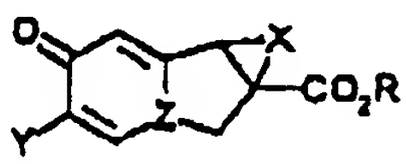
X = H, F, Cl, Br, OH, OR'
Y = H, F, Cl, Br, OH, OR'
Z = NH, NR", CH_2, O, S, SO, SO_2

VII



X = H, F, Cl, Br, OH, OR'
Y = H, F, Cl, Br, OH, OR'

VIII



X = CH_2, O
Y = H, F, Cl, Br, OH, OR'
Z = N, C-CH_3

IX

wherein Q,X,Y and Z are defined above for each series of compounds, and R, R' and R" are each independently alkyl groups (C_1-C_{20}); preferably lower alkyl groups (C_1-C_4). Moreover, the invention encompasses pharmaceutically acceptable salts of these compounds.

The compounds of the present invention can be synthesized in accordance with standard organic chemical techniques using readily/commercially available starting materials. Compounds of formula I are synthesized, for example, by oxidation of the appropriate m-hydroxyphenylaline derivative with periodate or other suitable oxidant. For example, I (X =Y= OH) is available from the commercially available DL-*threo*-β-(3,4-dihydroxyphenyl)serine (Sigma). Other m-hydroxyphenylalines are available by transformations of 3-hydroxy-4-substituted cinnamic acids.

Compounds of formula II (Z = NH or NR, X = OH) are synthesized by reduction of compounds of formula I with dithionite or other suitable reducing agent, followed, in the case of Z = NR, by alkylation of the nitrogen with an R-halide. Compounds of formula II (Z = CH_2) are synthesized by transformations of appropriately 5,6-substituted indene or 1- or 2-indanone derivatives. Compounds of formula II (Z = O or S) are synthesized by transformations of appropriately 5,6-substituted benzofuran or thianaphthene derivatives, respectively. Compounds of formula II (Z = SO or SO_2) are prepared by oxidation of II (Z = S) with hydrogen peroxide, peracids, or periodate under appropriate conditions.

Compounds of formula III are synthesized by treatment of corresponding compounds of formula II (Q = Cl or Br) with a suitable base to effect β-elimination of the halogen. Compounds of formula IV (Z = N, Y = OH or OR') are synthesized from kojic acid benzyl ether (2-hydroxymethyl-5-benzyloxy-4-pyranone) by reaction with β-alanine ethyl ester to give 2-hydroxymethyl-5-benzyloxy-4-pyridone-1-propionic acid ethyl ester; the hydroxymethyl group is oxidized to the aldehyde, 2-formyl-5-benzyloxy-4-pyridone-1-propionic acid ethyl ester, with chromium trioxide pyridine complex, and cyclization is affected by treatment with a base, such as lithium diisopropylamide, to give a compound of formula IV (Z = N, X = OH, Y = OCH_2Ph). From the latter, compounds of formula IV (X = OH) may be prepared by treatment with appropriate reagents for conversion of hydroxy to halogen (*e.g.*, diethylaminosulfur trifluoride, thionyl chloride, or thionyl bromide). For formula IV (Y = halogen) compounds, 2-formyl-5-(2-tetrahydropyranyl)oxy-4-pyridone-1-propionic acid ethyl ester, is prepared from 2-hydroxymethyl-5-(2-tetrahydropyranyl)oxy-4-pyranone by reaction with β-alanine ethyl ester, followed by oxidation with chromium trioxide pyridine complex. The product is then hydrolyzed in mild acid to 2-formyl-5-hydroxy-4-pyridone-1-propionic acid ethyl ester, which is treated with an appropriate dehydroxyhalogenation agent, such as diethylaminosulfur trifluoride, thionyl chloride, or thionyl bromide, to give 2-formyl-5-halogeno-4-pyridone-1-propionic acid ethyl ester. This product is then further converted, as in the case of Y = benzyloxy.

Compounds of formula IV (Z = C-CH_3, X = OH) are prepared from the corresponding 4-substituted-5-hydroxy-2-methylbenzaldehyde derivative by treatment with base to form a

phenoxide anion, followed by cyclization with an acrylic ester. Compounds of formula IV (Z = C-CH_3, X = F, Cl, or Br) are prepared by treatment of the appropriate compound of formula V (see below) with the corresponding hydrogen halide in a non-hydroxylic solvent; and subsequently treated with a lower alkoxide. This affords the compounds of formula V (Z = C-CH_3, X or OR'), which alternatively are synthesized by treatment of compounds of formula IV (Z = C-CH_3, X = OH) with an alkyl halide and a non-nucleophilic base.

Compounds of formula V (Z = N) are synthesized by treatment of the corresponding compounds of formula II (X = Cl or Br) with a suitable base to effect β-elimination of the halogen. Compounds of formula V (Z = C-CH_3) are prepared from the corresponding 4-substituted-5-hydroxy-2-methylbenzaldehyde derivative by treatment with base to form a phenoxide anion, followed by cyclization with diethyl methylenemalonate to form a derivative of the corresponding compound of formula IV (Z = C-CH_3, R = Et, X = OH) bearing a second carbethoxy group geminal to the carboxylic group depicted in the structures of formula IV. This product is then subjected to hydrolysis, decarboxylation and elimination, preferably under acidic conditions, to form compounds of formula V (Z = C-CH_3, R = H), which may be re-esterified.

Compounds of formula VI are synthesized from the corresponding 6,7-substituted quinoline derivative by treatment with benzoyl chloride and sodium cyanide to give the 1-benzoyl-2-cyano-1,2-dihydro derivative, followed by hydrogenation to give the 1-benzoyl-2-cyano-1,2,3,4-tetrahydro derivative. This product is hydrolyzed under basic or acidic conditions to give the corresponding 6,7-substituted-1,2,3,4-tetrahydroquinoline-2-carboxylic acid, which may then be converted to an ester, such as the methyl ester. Dehydrogenation at the 1,2-position is effected by treatment with, for example, an oxidant, a halogenation agent, or a sulfonylation agent, followed by base.

Compounds of formula VII are synthesized from corresponding compounds of formula III by treatment with an appropriate carbenoid or methylene transfer reagent, such as by treatment with diazomethane, or by treatment with diiodomethane in the presence of zinc dust, or by treatment with bromoform in the presence of potassium tert-butoxide followed by debromination of the resulting bibromomethano derivative (*e.g.*, using zinc in acetic acid).

Compounds of formula VIII are synthesized from compounds of formula III (Z = CH_2) by treatment with an epoxidizing agent, such as *m*-chloroperbenzoic acid. Compounds of formula IX (X = O) are likewise synthesized from compounds of formula V by treatment with an epoxidizing agent, such as *m*-chloroperbenzoic acid. Compounds of formula IX (X = CH_2) are synthesized from compounds of formula V under suitable methylene transfer conditions, such as, by treatment with diazomethane.

Pharmaceutical Formulations

The compounds identified by the inventive screening assay which inhibit MIF tautomerase activity are pharmaceutical compounds having therapeutic activity for inhibiting MIF immunomodulatory activity. The identified compounds of the present invention have

utility in pharmacological compositions for the treatment of various conditions involving cytokine-mediated toxicity, which include, but are not limited to shock, inflammatory diseases, graft-versus-host disease, and autoimmune diseases. An identified compound can be administered to a human patient, by itself, or in pharmaceutical compositions where it is mixed with suitable carriers or excipients at doses to treat or ameliorate various conditions involving cytokine-mediated toxicity, which include, but are not limited to shock, inflammatory diseases, graft-versus-host disease, and autoimmune diseases. A therapeutically effective dose further refers to that amount of the compound sufficient to inhibit the biological activity of MIF *in vivo*. Techniques for formulation and administration of the compounds of the instant application may be found in "*Remington's Pharmaceutical Sciences*" Mack Publishing Co., Easton, PA, latest edition.

Suitable routes of administration include, for example, oral, rectal, inhalation, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. Alternatively, one may administer the therapeutically compound in a local rather than systemic manner, for example, via injection of the compound directly at the site of inflammation, often in a depot or sustained release formulation. Furthermore, one may administer the compound in a targeted drug delivery system, for example in a liposome coated with an anti-MIF receptor antibody. The liposomes will be targeted to and taken up selectively by cells expressing MIF receptor.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, such as, by means of conventional mixing, dissolving, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes. Such pharmaceutical compositions may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into pharmaceutical preparations. Proper formulation is dependent upon the route of administration chosen.

For parenteral injection, the inventive pharmaceutical compositions may be formulated in aqueous solutions, preferably in physiologically compatible buffers, such as Hank's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. For oral administration, the inventive pharmaceutical compositions can be formulated by combining the therapeutically active compounds with pharmaceutically acceptable carriers. Such carriers enable the inventive pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral administration to a patient. Pharmaceutical preparations for oral use can be obtained as a solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose,

mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate. Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration. For buccal administration, the inventive pharmaceutical compositions may take the form of tablets or lozenges formulated in conventional manner. The inventive pharmaceutical compositions may also be formulated in rectal compositions, such as suppositories or retention enemas (*e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides).

For administration by inhalation, the inventive pharmaceutical compositions are delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant (*e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas). In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the inventive pharmaceutical compositions and a suitable powder base such as lactose or starch.

The inventive pharmaceutical compositions may be formulated for parenteral administration by injection, such as, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, such as, in ampoules or in multi-dose containers, with an added preservative. The inventive pharmaceutical compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulary agents such as suspending, stabilizing and/or dispersing agents. Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl

oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, such as, sterile pyrogen-free water, before use.

The inventive pharmaceutical compositions may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various of sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include, but are not limited to, calcium carbonate, calcium phosphate, sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Many of the MIF inhibiting compounds of the invention may be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts may be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc.; or bases. Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms. Examples of pharmaceutically acceptable salts, carriers or excipients are well known to those skilled in the art and can be found, for example, in *Remington's Pharmaceutical Sciences*, 18th Edition, A.R. Gennaro, Ed., Mack Publishing Co., Easton, PA, 1990. Such salts include, but are not limited to, sodium, potassium, lithium, calcium, magnesium, iron, zinc, hydrochloride, hydrobromide, hydroiodide, acetate, citrate, tartrate, malate salts, and the like.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount effective to prevent development of or to alleviate the existing symptoms or treat the

underlying disease of the subject being treated. The therapeutically effective dose concentration can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC_{50} as determined in cell culture (that is, the concentration of the compound which achieves a half-maximal inhibition of the MIF activity). A therapeutically effective dose refers to that amount of the inventive pharmaceutical composition that results in amelioration of symptoms or a prolongation of survival in a patient. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical, pharmacological, and toxicological procedures in cell cultures or experimental animals. The dose ratio between toxic and therapeutic effects is the therapeutic index. Compounds which exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. The amount of composition administered will be dependent on the patient being treated, on the patient's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

Example 1

This example illustrates inhibition of MIF tautomerase activity with glutathione. A reaction mixture was prepared containing 0.5 mM of the MIF-substrate L-dopachrome methyl ester by oxidation of L-DOPA methyl ester with periodate. A 1 mM solution of L-DOPA methyl ester was prepared in Reaction Buffer (10 mM sodium phosphate, pH 6, containing 0.5 mM EDTA). L-DOPA methyl ester was oxidized to colored L-dopachrome methyl ester by adding 10% ($^{v}/_{v}$) of a 20 mM aqueous solution of potassium periodate, following a 1:2 dilution with Reaction Buffer. After approximately 10 minutes, the color remained stable and the substrate solution was ready for use in the assay.

The test compound, reduced glutathione, was added in concentrations of 0.1 mM, 0.5 mM, 1.0 mM, and 2.0 mM to separate cuvettes containing the substrate solution. Approximately 100 ng of purified recombinant MIF was added to each cuvette. The absorbance at 475 nm of each sample cuvette was measured over the first 20 seconds to 2 minutes. The initial rate of change in absorbance, indicating reaction rate or velocity, was recorded. This decrease in absorbance following addition of recombinant MIF was used to determine the tautomerase activity of recombinant MIF in the presence of glutathione. Results are shown in Table 2 below.

TABLE 2

Glutathione in Assay [mM]	% Inhibition
0	0%
0.1	14%
0.5	69%
1.0	95%
2.0	100%

At 0.5 mM to 2.0 mM concentrations, glutathione was an effective inhibitor of MIF tautomerase activity.

Example 2

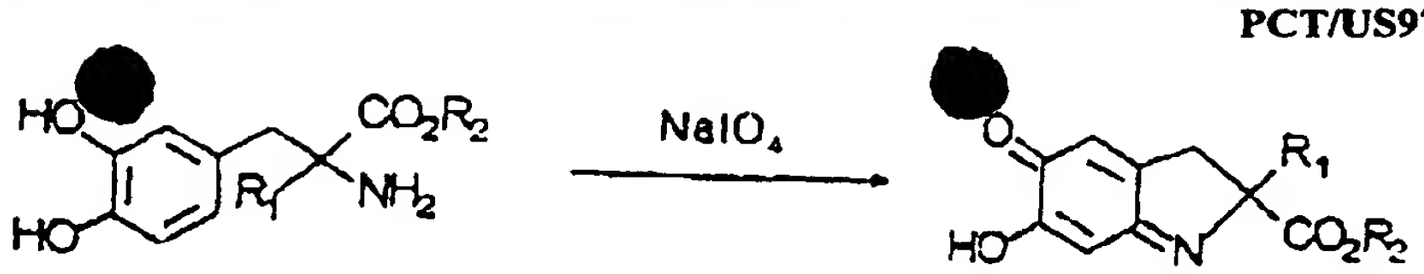
This example illustrates the influence of the native MIF amino terminus primary sequence on MIF tautomerase activity. An amino-terminal mutation of murine MIF was expressed in *E. coli* and assayed for tautomerase activity. The coding region of murine MIF was mutagenized by the addition of a three amino acid extension sequence: methionine-aspartate-serine, to the native amino terminus. The altered coding region of murine MIF was expressed in *E. coli* and the expressed protein was purified and assayed for MIF tautomerase activity as described above. Expression of this altered gene resulted in an MIF protein entirely lacking tautomerase activity. The expression of human MIF bearing an unaltered, native amino-terminus in the same *E. coli* system resulted in a highly active MIF, the tautomerase activity of which could be detected even in crude bacterial lysates, without purification.

Additional mutations of the MIF primary sequence also destroyed MIF tautomerase activity. Specifically, a deletion mutant eliminating the N-terminal proline of MIF caused loss of all tautomerase activity. A substitution of the N-terminal proline (by serine) caused loss of all tautomerase activity. Moreover, C-terminal truncation mutants 1->104 and 1->110 had no tautomerase activity.

The complete loss of MIF tautomerase activity by amino-terminal extension or mutation suggests a crucial influence of this region on MIF tautomerase activity.

Example 3

This example illustrates an ability of compounds 2b [L-3,5-dihydro-6-hydroxy-2-methyl-5-oxo-2H-indole-2-carboxylic acid], 3b [D/L-3,5-dihydro-6-hydroxy-2-methyl-5-oxo-2H-indole-2-carboxylic acid], 4b [L-3,5-dihydro-6-hydroxy-2-methyl-5-oxo-2H-indole-2-carboxylic acid methyl ester], and 5b [D/L-3,5-dihydro-6-hydroxy-2-methyl-5-oxo-2H-indole-2-carboxylic acid methyl ester] to inhibit MIF tautomerization activity *in vitro*.



a: DOPA deriv

b: Dopachrome deriv

	D/L	R_1	R_1
1a, 1b:	L	H	CH_3
2a, 2b:	L	CH_3	H
3a, 3b:	DL	CH_3	H
4a, 4b:	L	CH_3	CH_3
5a, 5b:	DL	CH_3	CH_3

The general method of performing an assay for MIF dopachrome tautomerase activity begins with oxidation of a percursor of the MIF-substrate (*e.g.*, DOPA-related Compound 1a [L-3-(3,4-dihydroxyphenyl) alanine methyl ester] above) and, if required, a similar oxidation of precursors of the test inhibitors (*e.g.*, the DOPA-related Compounds 2a [L-3-(3,4-dihydroxyphenyl)-2-methyl alanine], 3a [D/L-3-(3,4-dihydroxylphenyl)-2-methyl alanine], 4a [L-3-(3,4-dihydroxyphenyl)-2-methyl alanine methyl ester] and 5a [D/L-3-(3,4-dihydroxyphenyl)-2 methyl alanine methyl ester] above). This oxidation of each precursor compound (with, for example, sodium periodate) generates a corresponding orange-colored dopachrome derivative; specifically, a preferred MIF-substrate (Compound 1b above) and the test inhibitors (Compounds 2b, 3b, 4b, and 5b above). Upon addition of MIF, the MIF-substrate (Compound 1b [L-3,5-dihydro-6-hydroxy-5-oxo-2H-indole-2-carboxylic acid methyl ester]) undergoes tautomerization, resulting in a colorless product. In the presence of the test inhibitors (Compounds 2b, 3b, 4b, and 5b), decolorization of MIF-substrate (Compound 1b) was retarded. Test compounds 2b, 3b, 4b, and 5b themselves were unreactive in this tautomerization reaction; that is, they were not tautomerized and correspondingly decolorized by MIF dopachrome tautomerase activity if they were substituted for the MIF-substrate in this assay method.

In a preferred MIF dopachrome tautomerase activity assay procedure, the DOPA-related precursors (Compounds 1a, 2a, 3a, 4a, and 5a above) were prepared as 10 mM stock solutions in Assay Buffer (10 mM sodium phosphate, pH 6.0). About 10 minutes before the tautomerization assay, 1.0 ml of each stock solution was diluted with 8 ml of Assay Buffer, and 1.0 ml of sodium periodate stock solution (20 mM in water), was added to initiate the oxidation of the diluted precursors, thus generating each respective dopachrome derivative (*i.e.*, Compounds 1b, 2b, 3b, 4b, and 5b) at a final concentration of about 1 mM. To perform the actual tautomerization assay, 0.5 ml of the 1 mM solution of the MIF-substrate (*i.e.*, Compound 1b) was mixed with 0.5 ml of Assay Buffer (no inhibitor control reaction) or 0.5 ml of the 1 mM dilution of the test inhibitor (*e.g.*, Compounds 2b, 3b, 4b, or 5b), followed by

addition of 10 ml of recombinant MIF solution from a 20 mg/ml stock. The decrease in absorbance at 475 nm in 1 minute was measured as an index of tautomerase activity, and the percent inhibition of this decrease in the presence of Compounds 2b, 3b, 4b and 5b was calculated from the spectrophotometric data by standard methods.

The results of this assay are shown in the table below:

TABLE 3

Inhibitor	% Inhibition of MIF Tautomerase Activity
Compound 2b	35
Compound 3b	29
Compound 4b	34
Compound 5b	45

When present at the same concentration as the MIF-substrate, test inhibitor Compounds 2b, 3b, 4b, and 5b each inhibited MIF tautomerization activity, as measured by the inhibition of the decrease in dopachrome-specific absorbance. These results indicate that abstraction of the α-hydrogen of the dopachrome-related MIF-substrate is a critical step in the MIF-catalyzed tautomerization, and that test compounds lacking this hydrogen atom (*e.g.*, those with an α-methyl substitution) are effective inhibitors of MIF dopachrome tautomerase activity.

Example 4

This example illustrates inhibition of MIF tautomerization activity by glycation of lysine residues. Upon prolonged exposure to glucose or another reducing sugar, *in vivo* or *in vitro*, proteins and other amino-containing biomolecules becomes spontaneously modified by covalently attached sugar-derived adducts (Bucala and Cerami, *Adv. Pharmacol.* 23:1-34, 1992). The chemistry of this process is broadly known as the Maillard reaction, wherein susceptible protein amino groups, such as the peptide amino-terminus and the ε-amino moieties of lysine residues, for example, first condense with a reactive carbonyl moiety of a reducing sugar to form a readily reversible Schiff base. This initial adduct can spontaneously rearrange to generate an Amadori product (or Heyns product, depending on the specific sugar involved), which is stable.

Purified, recombinantly-produced human MIF protein was glycated by incubation in aqueous buffer in the presence of 0.5 M glucose, typically for 6 weeks in the dark at 37 °C under a nitrogen atmosphere to ensure complete glycation of susceptible residues (although other spontaneous glycation conditions will serve to glycate the protein without departing from the essence of the invention) and tested for D-dopachrome tautomerase activity by the method described herein. This glycated or AGE-modified MIF ("AGE-MIF") showed no D-dopachrome tautomerase activity. The AGE-MIF was recovered and analyzed by matrix-assisted laser desorption ionization mass spectroscopy and determined to have a mass

consistent with three-fold Amadori product modification of the protein; that is, there was a mass excess of approximately 3 x 162 (the mass excess of a single Amadori product).

There are three lysine residues distributed within the primary sequence of human MIF. Since recombinantly-produced MIF protein begins with the second amino acid in the primary sequence, proline (because the normal initial vertebrate methionine residue is typically clipped off by the expressing bacteria), there is no free amino group associated with the amino-terminus of the protein. Taken together, this indicates that modification of side-chain amino groups of amino acids within the MIF peptide sequence can eliminate MIF D-dopachrome tautomerase activity. Thus, compounds that cause the modification of lysine residues of MIF are useful as inhibitors of MIF bioactivity, including, without limitation, the inflammatory and immunomodulatory activities of MIF.

Example 5

This example illustrates a periodate-free assay system to measure tautomerase activity of MIF. The tautomerase assay requires generation of fresh L-dopachrome methyl ester (L-DCME) from L-dopa methylester (L-DME) by chemical oxidation with excess periodate. Large amounts of oxidizing reagent periodate are required because no further purification steps are used. Moreover, remains of periodate further increase the instability of the substrate which requires the preparation of fresh substrate solutions, and periodate may also react with the test compounds. Therefore this example shows a method to remove remaining periodate and its reaction product iodate from the assay mixture and concentrate the substrate as a preferred embodiment of the inventive assay method.

After the formulation of L-DCME from L-DME and periodate, the substrate mixture is subject to reversed phase chromatography with C18-coated silica beads. Periodate is removed by flushing with deionized water. The substrate is eluted from the column with pure methanol to yield a methanolic solution of L-DCME that can be stably stored at -70 °C for at least several months.

Equal volumes of aqueous solutions of L-dopa methyl ester (L-DME) (4 mM) and sodium periodate (8 mM) were mixed and incubated for 5 min. The remaining periodate was removed from the deeply colored L-dopachrome methyl ester by chromatography over a self-packed 10 ml C18 reverse phase column using a vacuum device. The column was flushed three times with deionized water (30 ml) and the L-dopachrome methyl ester (DCME) was eluted with methanol. The methanolic eluate was stable at -70 °C for at least several months.

Using the above-noted improved reagents, a dopachrome tautomerase assay can mix 1 ml of buffer A (0.2% Tween in 25 mM potassium phosphate pH 6.0) of buffer B (500 μM EDTA in 25 mM potassium phosphate pH 6.0) with 10-30 μl of the substrate L-DCME concentrate (starting $E_{475nm} \approx 1\text{-}1.4$). After monitoring the background rate, MIF is added (typically 0.05 to 0.5 μg MIF). The background and MIF-catalyzed reactions are monitored at 475 nm on a spectrophotometer.

What is claimed is:

1. A screening assay for compounds that inhibit the biological activity of MIF, comprising:

(a) preparing a reaction mixture of MIF and a MIF-substrate in the presence and absence of a test compound; and

(b) detecting tautomerization of the MIF-substrate,

whereby a decrease in tautomerization of the MIF-substrate indicates the ability of the test compound to inhibit MIF activity.

2. The screening assay of claim 1 wherein the MIF-substrate comprises D-dopachrome, D-dopachrome-methyl ester, or L-dopachrome-methyl ester.

3. The screening assay of claim 2 wherein tautomerization of the MIF-substrate is detected colorimetrically or spectrophotometrically.

4. A method for inhibiting immunomodulatory activity of MIF in a mammal, comprising administering an effective amount of an agent that inhibits the tautomerase activity of MIF.

5. The method of claim 4 wherein the agent inhibits the MIF-catalyzed tautomerization of D-dopachrome, D-dopachrome-methyl ester, or L-dopachrome-methyl ester.

6. The method of claim 4 wherein the agent is used to treat disorders selected from the group consisting of shock, inflammatory diseases, graft-versus-host disease, and autoimmune disease.

7. The method of claim 4 wherein the agent is used to treat inflammatory diseases.

8. The method of claim 7 wherein the agent is administered in conjunction with glucocorticoid steroid therapy.

9. The method of claim 4 wherein the agent is an organic compound, a protein or a peptide.

10. The method of claim 4 wherein the agent is a D- and L-forms of α-methyl dopachrome methyl ester.

11. The method of claim 4 wherein the agent is selected from the group consisting of compounds 2b [L-3,5-dihydro-6-hydroxy-2-methyl-5-oxo-2H-indole-2-carboxylic acid], 3b [D/L-3,5-dihydro-6-hydroxy-2-methyl-5-oxo-2H-indole-2-carboxylic acid], 4b [L-3,5-dihydro-6-hydroxy-2-methyl-5-oxo-2H-indole-2-carboxylic

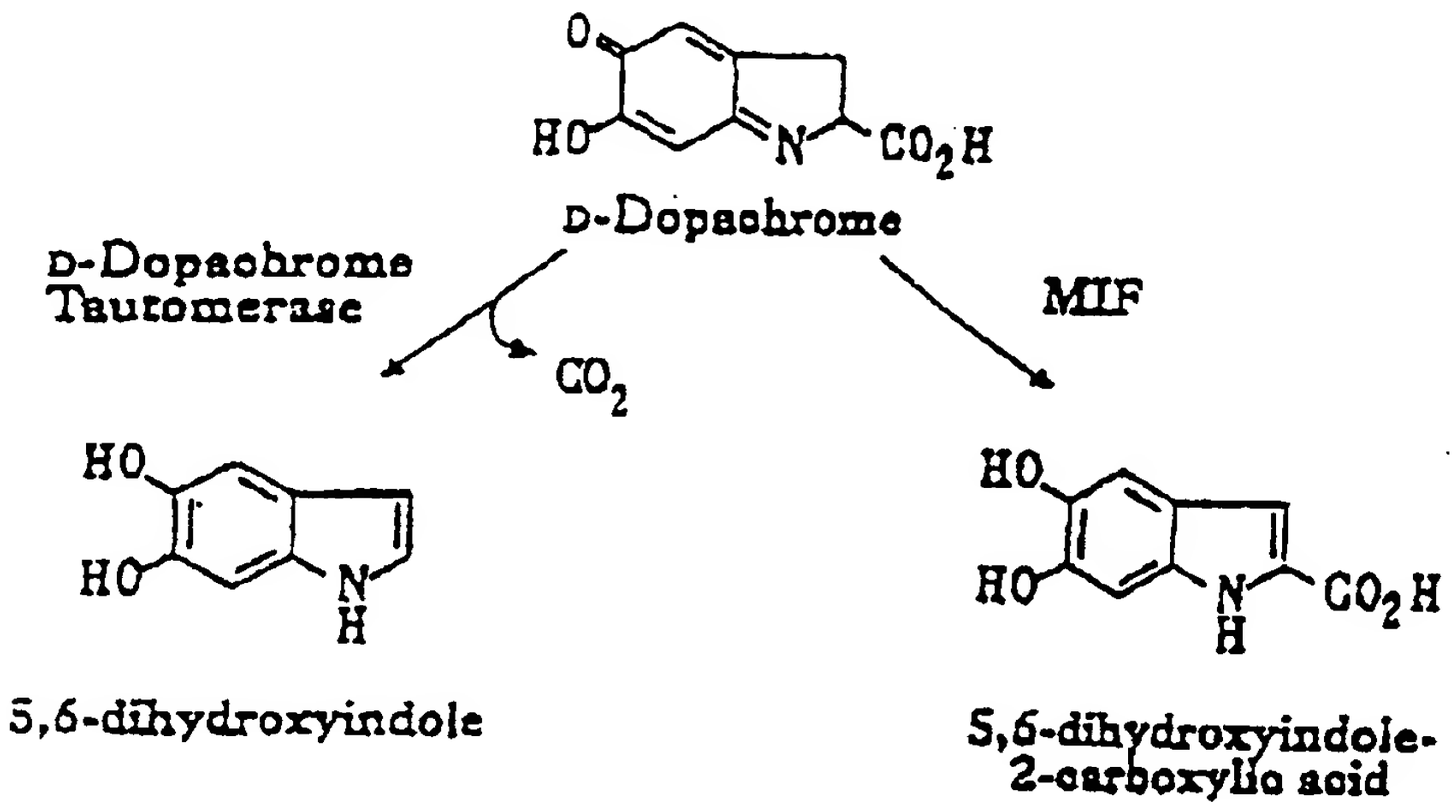


FIG. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/02448

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 43/34, 43/40; A61K 38/06, 31/435; C07K 5/00
US CL :514/415, 201, 277.4; 530/331

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/415, 201, 277.4; 530/331

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, WPIDS, SCISEARCH, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ROSENGREN, E. et al. The Immunoregulatory Mediator Macrophage Migration Inhibitory Factor (MIF) Catalyzes a Tautomerization reaction. Molecular Medicine. January 1996, Vol. 2, No. 1, pages 143-149, see entire document.	1-11
Y	AROCA et al. Specificity of Dopachrome Tautomerase and Inhibition by Carboxylated Indoles. Biochem. J. 1991, Vol. 277, pages 393-397, see entire document.	1-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
07 APRIL 1997	25 APR 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PADMASHRI PONNALURI gab for Telephone No. (703) 308-0196

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